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Analysis of secretory immunoglobulin A in human saliva by laser-induced fluorescence capillary electrophoresis

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Abstract

The utility of capillary electrophoresis (CE) has been demonstrated for the analysis of secretory immunoglobulin A (sIgA) in human saliva. The amount of sIgA in saliva correlates with immune status. For detecting salivary sIgA, laser-induced fluorescence was conducted in this report for signal amplification. sIgA and anti-sIgA antibody were labeled with cyanine fluorescence (Cy5) for competitive immunoassay and non-competitive analysis, respectively. Cy5 was excited by He–Ne laser with a wavelength of 635 nm, with maximum emission at 670 nm. Migration time during electrophoresis depended on whether sIgA–Cy5 was mixed with antibody or anti-sIgA–Cy5 mixed with sIgA to form Ag–Ab complex. The results indicated that CE competitive immunoassay was effective for analyzing serum sIgA, but not for salivary sIgA. However, salivary sIgA can be analyzed by complex formation assay. The peak area of the complex was proportional to the amount of sIgA added. A standard linear regression curve was generated using purified sIgA. From this standard curve, the amount of sIgA from saliva of either normal or immunocompromised patients can be calculated from the Ag–Ab complex peak area.

Keywords: Immunoglobulin A

1. Introduction

Saliva is secreted from three pairs of glands in the buccal cavity. The parotid gland is the major source of saliva, secreting more than 70% of total saliva. The composition of saliva includes proteins, carbohydrates, hormones and antibodies, especially secretory immunoglobulin A (sIgA). Secretory immunoglobulin A is the dominant immunoglobulin in external mucosal secretions such as oral, respiratory, and intestinal cavities, and is often characterized as a component of the immune system ''first-line defense'' against pathogenic microorganisms, viruses, and bacteria [1]. The sIgA molecule is a dimeric IgA which contains two units of IgA. The two units of IgA are conjugated by a J chain to form sIgA, which then migrates to the surface of oral and respiratory mucosa. The dimeric structure confers a higher resistance to the enzymes which are abundant in the mucus. The amount of sIgA secreted is related with age. At birth, levels of sIgA are undetectable, but there is a consistent increase until age 7. Levels

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remain constant through adolescence to mid-life, and then decline during old age. No gender differences in sIgA levels have been reported. Several investigators have reported that levels of sIgA are not directly reflected to serum levels of sIgA [2–5]. Low levels of sIgA are considered a risk factor for upper respiratory infections in children and the elderly [2–4]. In addition, lower levels of sIgA have been reported to increase risk for periodontal disease and caries [6,7]. Other investigators have found that lower levels of sIgA may link academic stress and emotional stress [8,9]. The current methods used to assess the amount of sIgA in saliva are either radioimmunodiffusion or enzyme-linked immunosorbent assay (ELISA). These methods are inaccurate and the processes are cumbersome. In this report, the capillary electrophoresis (CE) equipped with laser-induced fluorescence (LIF) was used to assess the amount of sIgA in saliva. Advantages of CE with respect to other analytical techniques, including minimal required sample volume, rapid analysis, higher resolution, and low cost, have made this technique ideal for analysis of a number of endogenous and exogenous substances present in biological fluids. The different modes of CE have been coupled to different detection techniques, such as UV absorbance, electrochemical, mass spectrometry and LIF in order to detect different natural and molecular size-separated analytes. In this report, analyses were performed by preparing purified human sIgA or anti-human sIgA labeled with a bisfunctional dye, for competitive immunoassay or complex formation assay respectively. The quantitative analyses were applied to either healthy control or patients suffering from recent influenza or in the recovery period.

2. Experimental

2.1. Materials

Boric acid, acetic acid, sodium hydroxide, CHAPS (3-[3-cholamidopropyl dimethylammoniol]-1-propanesulfonate), goat anti-human IgA, phosphoric acid, sodium carbonate and sodium hydrogencarbonate were purchased form Sigma (St. Louis, MO, USA). Purified human secretory IgA was purchased from ICN (Irvine, CA, USA) and Cy5 conjugated affinity purified secondary antibody were purchased from Chemicon (Temecula, CA, USA). Cy5 bisfunctional dye PA25000 and Sephadex PD-10 columns (G-25) were purchased from Amersham Pharmacia (Piscataway, NJ, USA).

2.2. Apparatus

LIF detection was performed on a P/ACE system Model 2100 equipped with a He–Ne laser detector (Beckman Instruments, Fullerton, CA, USA) controlled by System Gold software (San Ramon, CA, USA). Laser was generated from a 635 Laser Module (Beckman Instruments) and connected with capillary cartridge by fiberoptic cable. When fluorescent (Cy5) passed the detection window, it can be excited to induce emission. The emission light was collected and through a long-pass filter (>675 nm). Capillary columns were purchased from Polymicro Technologies (Phoenix, AZ, USA). The dimensions of capillary were 27 cm×50 μm I.D. without coating. The field strength for electrophoresis was 555 V/cm. The sample was injected by positive pressure (0.5 p.s.i. for 10 s; 1 p.s.i.=6894.76 Pa).

2.3. Buffer solution

Boric acid based buffer (isoelectric point, pI= 9.24) was used for LIF analysis. The buffer was prepared by adding 4.63 g of boric acid dissolved in 450 ml of degassed deionized distilled water. After completely dissolving, 0.5 g of CHAPS was added and the pH brought to 8.5 by adding NaOH (0.2 M). The final solution was then adjusted to a total volume of 500 ml, containing 150 mM boric acid, 1% CHAPS, pH 8.5. The solution was used as the running buffer after passing through a 0.45-μm filter membrane.

2.4. Sample pretreatment

Each saliva sample was placed on ice immediately after collection in the morning from a donor who was fasting overnight. The sample from each donor was divided into two groups. One group was immediately acidified to pH 4.5 with 2 M acetic acid, then boiled for 2.5 min to halt proteolytic degradation. The sample was then left on ice for 20 min, centrifuged for 30 min at 13 000 rpm in a Kubota

5200 rotor (Tokyo, Japan). The supernatant fraction was subjected to CE analysis. The other group was analyzed directly after centrifugation (30 min at 13 000 rpm).

2.5. Running conditions

The newly installed capillary was rinsed with 0.2 *M* NaOH for 20 min and followed by rinsing with distilled–deionized water (2D H_2O) for 30 min, and then was equilibrated by running buffer for another 60 min. Sample was injected by positive pressure injection (0.5 p.s.i.) for 10 s. The electrophoretic field strength was fixed at 555 V/cm and the current was 10 μA. Between each run, the capillary was regenerated with 0.2 *M* NaOH, 2D H_2O and running buffer for 10 min each.

2.6. Fluorescence labeling

A total of 5 mg of purified sIgA was dissolved in 1 ml of sodium carbonate, sodium hydrogencarbonate solution (0.1 *M*, pH 9.3). By adding 0.5 mg of Cy5 and completely mixing, the mixture was incubated at room temperature for 60 min. For removing the unbound Cy5, the mixture was then passed through a Sephadex PD-10 column chased by 0.15 *M* phosphate-buffered saline (PBS). The conjugate was collected and identified by CE. This Cy5 labeled sIgA was used for CE immunocompetitive analysis. The same processes were used for anti-IgA antibody labeling. The labeled antibody was ready for the non-competitive analysis.

3. Results and discussion

3.1. Optimization of the running buffer pH for antigen–antibody formation

To verify the optimal running buffer, the conjugated sIgA–Cy5 was mixed with antibody and incubated for 30 min. The mixture was subjected for the capillary electrophoresis. The pH of running

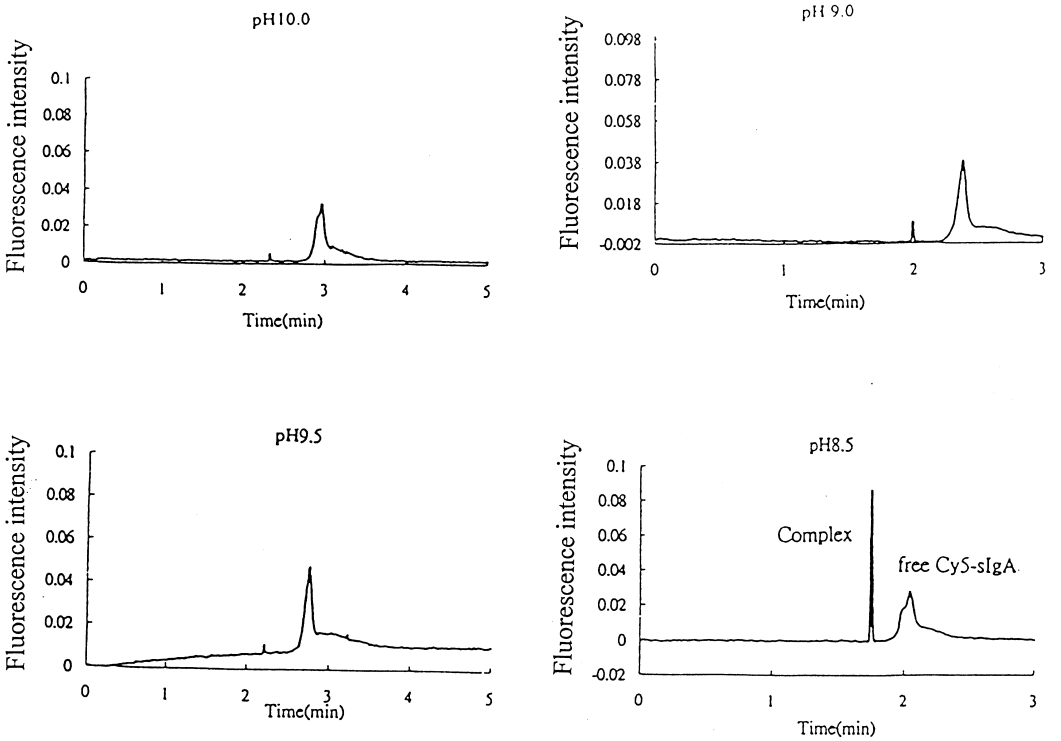

Fig. 1. The pH boric acid based buffer (150 m*M* boric acid with 1% CHAPS) was adjusted to 10.0, 9.5, 9.0 and 8.5 for the Ag–Ab complex formation. The results showed that the optimal pH for complex formed is 8.5 (bottom right).

buffer was adjusted from the pH 8.5 up to pH 10.0 by 1 *M* NaOH. The results, shown in Fig. 1, indicate that maximal Ag–Ab formation occurred at running buffer pH 8.5. For improving the resolution, several zwitterions were tested. We found that 1% of CHAPS with 150 m*M* boric acid at pH 8.5 provided the best resolution for separating sIgA–Cy5 and Ag–Ab complex. Under the experimental conditions, we noted that the higher pH, the lower the interaction between antigen and antibody. The sharp peak showed in the electropherogram seemed to indicate that the Ag–Ab may have a tendency to aggregate. However, the aggregation did not form particles large enough to block the capillary and the migration time. The relative standard deviation (RSD) of migration time of the complex is 0.68% which is fairly consistent.

3.2. On-column immunocompetitive analysis for sIgA

In this experiment, a set amount of anti-sIgA was added into the solution containing Cy5 conjugated sIgA (Cy5–sIgA). The results showed that when

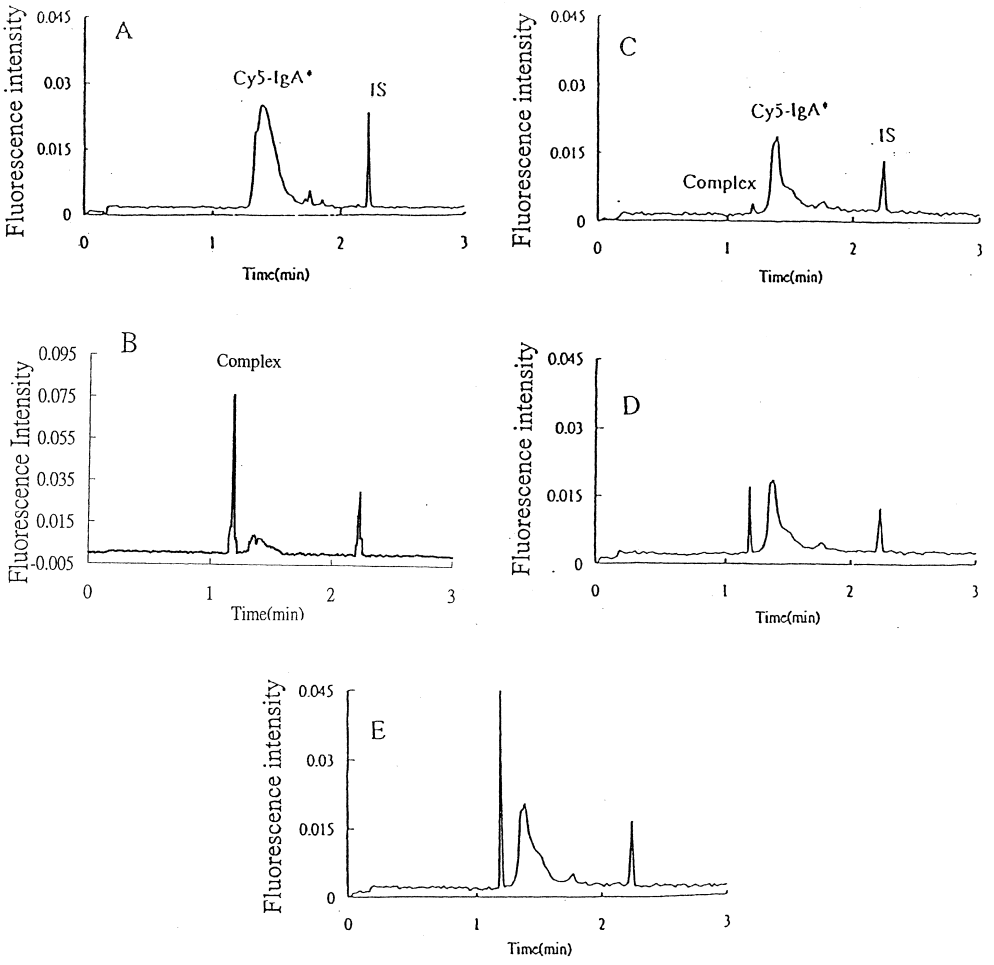

Fig. 2. Immunocompetitive assay: (A) Cy5–sIgA (20 μg/20 μl) alone, (B) (A) mixed with 10 μg/10 μl anti-sIgA, (C) (B) mixed with 3.15 μg/20 μl sIgA, (D) (B) mixed with 2.22 μg/20 μl sIgA, (E) (B) mixed with 1.18 μg/20 μl sIgA. The peak area of Ag–Ab complex is inverted proportional to the amount of sIgA added. I.S. represented the internal standard which is free Cy5 (10^{-3} μg/μl) mixed with sample.

10 μl of anti-sIgA (1 mg/ml) was added to 20 μl Cy5–sIgA, it induced the maximal amount of complex. A sharp and high complex peak with a minimal broad Cy5–sIgA peak are shown in the electropherogram (Fig. 2B). The mixture was used for generating the standard curve for the quantitative immunocompetitive assay by adding various amounts of purified anti-sIgA into it. To standardize

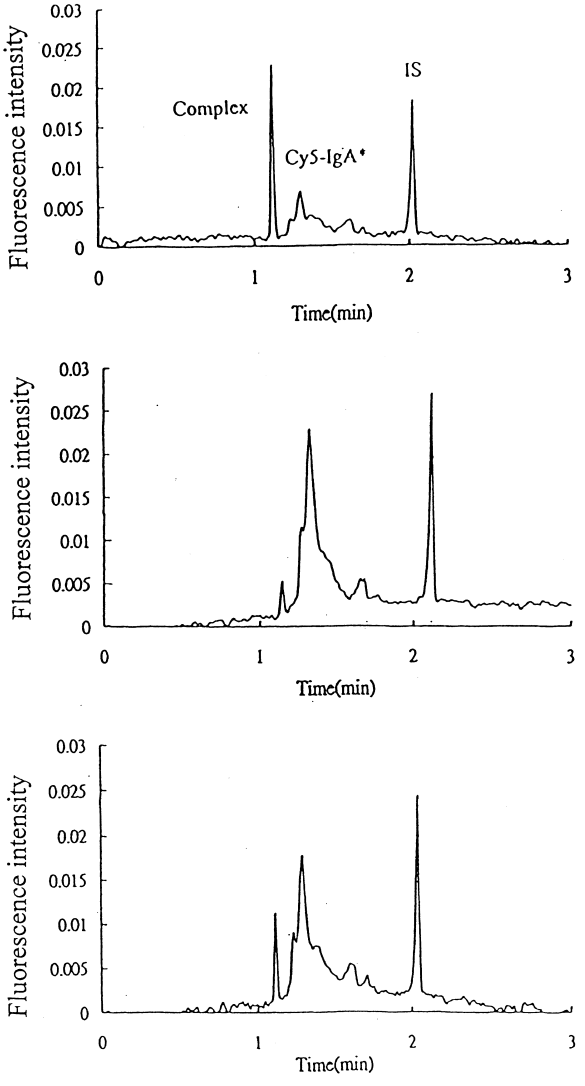

Fig. 3. Analysis of serum IgA by immunocompetitive assay. The upper panel is the assay mixture which contains 20 μl Cy5–sIgA and 25 μl anti-sIgA Ab. Middle panel is 5× diluted serum was added in the assay mixture. Bottom panel is 10× diluted serum was added in the assay mixture.

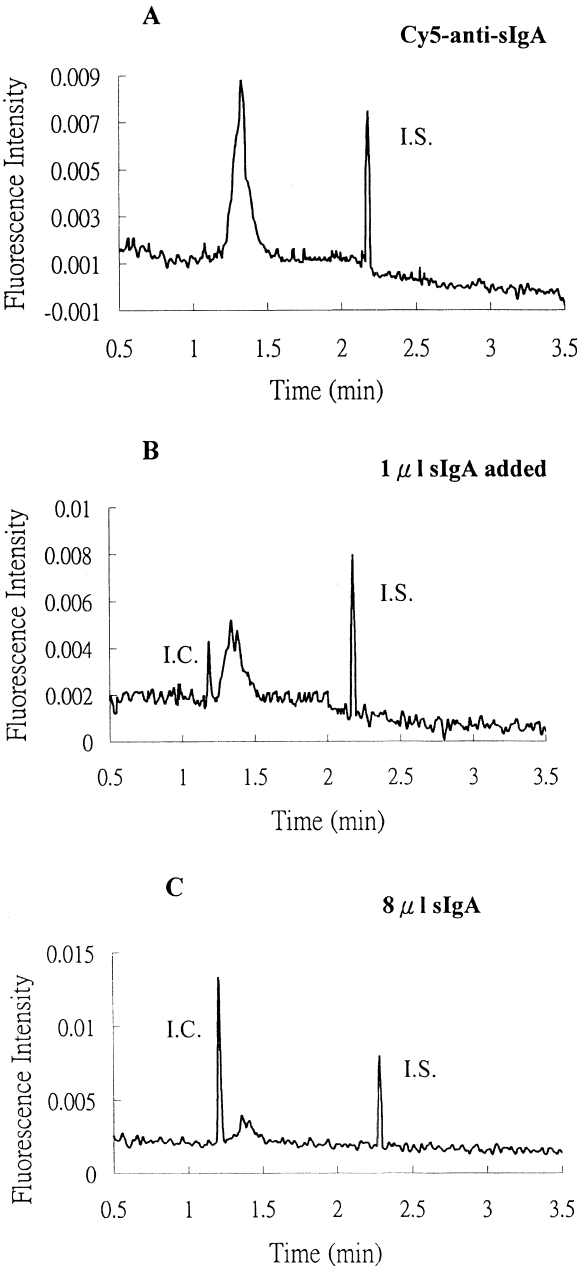

Fig. 4. Cy5 labeled anti-sIgA Ab was used for detecting saliva sIgA. When sIgA was added with Cy5 labeled Ab, a narrowed sharp peak complex appeared (migration time around 1.2 min) in ahead of Cy5–anti-sIgA peak and the peak area is proportional to the amount of diluted sIgA was added. (A) Cy5–anti-sIgA alone with 40 μl of PBS, (B) Cy5–anti-sIgA with 1 μl sIgA in 39 μl of PBS, (C) Cy5–anti-sIgA with 8 μl sIgA in 32 μl of PBS.

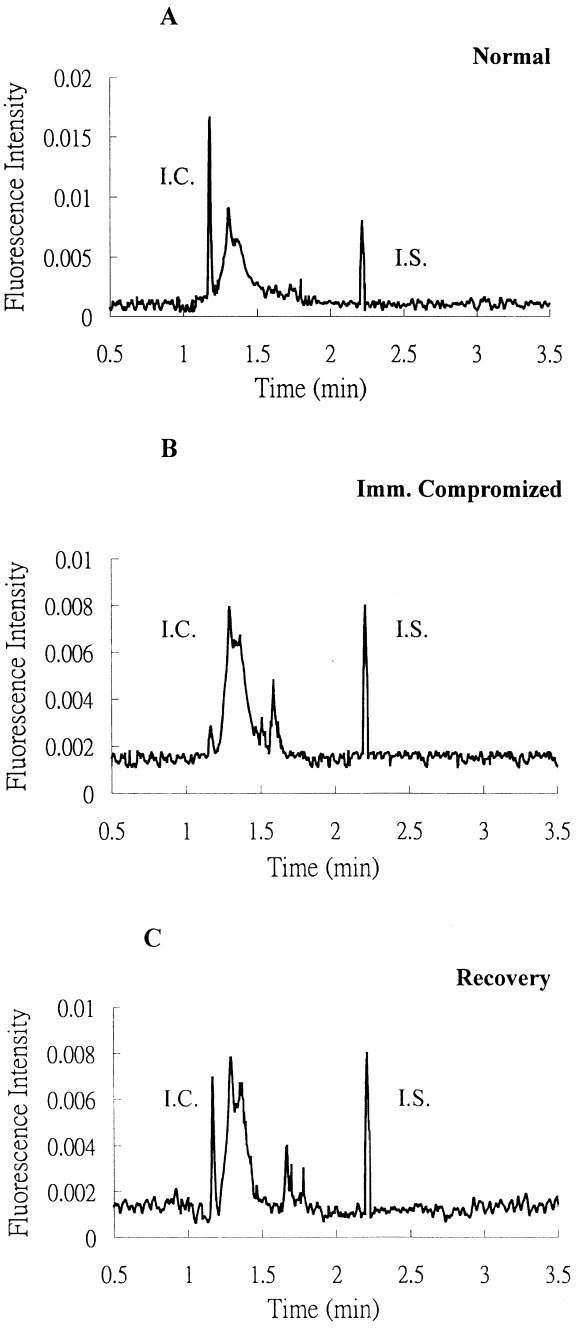

Fig. 5. Analysis of salivary sIgA by Ag–Ab formation assay. Three saliva samples, which include (A) normal healthy person, (B) immunocompromised patient (viral infected), and (C) person recovered from viral infection. The peak area of I.C. appeared an attenuated at the electropherogram from the immunocompromised patient saliva sample compared with either normal or recovered from common cold. I.C., immunocomplex.

the peak height and area, a fixed amount of free Cy5 (1 μl of 10^3 diluted Cy5) was added. Fig. 2C–E, shows that the complex peaks are inversely related with the amount of purified sIgA added into the mixture (C: 3.15 μg/20 μl, D: 2.22 μg/20 μl, E: 1.18 μg/20 μl). The linear regression curve showed that the R^2=0.9789, slope=−0.0105 and intercept=1.7744. This assay mixture was used for assessing salivary sIgA, however, the amount of sIgA seemed insufficient to form complexes, even as excess amounts of saliva were added. The same method was used for assessing serum sIgA. We found that the amount of sIgA in serum was enough to form complexes even in 10× diluted with PBS (Fig. 3). The antibody used in this experiment is anti-α chain. Anti-α chain not only binds with sIgA but also with IgA monomers. The concentration of serum IgA ranged from 0.1 to 1 mg/ml. In saliva however, it was only about 100-fold less than in serum. Therefore, the failure to detect salivary sIgA may be due to lack of sensitivity with this method. An alternative way to do the immunoassay in capillary electrophoresis is to label anti-sIgA antibody (Cy5–anti-sIgA) and then adding saliva containing sIgA. When sIgA is bound with Cy5–anti-sIgA, it migrates at a different speed, and the peak can be distinguished from the electropherogram. Fig. 4 shows that the Cy5–anti-sIgA alone mixed with 40 μl PBS (A), Cy5–anti-sIgA mixed with 1 μl (1 mg/ml) purified sIgA in 39 μl PBS, (B) to make the total volume of 60 μl and gradually increased the amount of sIgA from 2 to 4 and 8 μl (only 1 and 8 μl are shown in the figure), all samples were spiked with 4 μl of free Cy5 (1:2000 dilution) as an internal standard. The results showed that the complex formation is proportional to the amount of sIgA was added. The labeling procedures were followed identically as labeling to sIgA. However, the efficiency of labeling seemed inferior to the prior trial. The fluorescence intensity of the conjugate was less than 0.01 unit (arbitrary units) which cause a fluctuated baseline because of the small amount of conjugate. However, the small amount of conjugate may improve the sensitivity of the assay. For generating the standard curve, the concentration of added sIgA ranged from 1 to 8 μg/ml, which is the range of normal sIgA content in saliva. The peak area of complex and the internal standard were integrated by System Gold and the

Table 1
Three saliva samples from different immunity status were assessed by complex formation assay

	a	*b*	*a*/*b*=*y*	*x* (Concentration)
Normal stage	0.3788	0.26845	1.411063513	9.059654157
Immnocompromized patient	0.03066	0.27003	0.11354294	−0.195128818
Recovery stage	0.38298	0.26914	1.422976889	9.144628312

The ratio of I.C. peak and I.S. peak and the concentration of salivary sIgA were calculated from linear regression curve ($y=0.1402x+0.1409$).

ratio was calculated. The linear regression between sIgA concentration vs. peak area ratio of complex/Cy5 was calculated as the correlation coefficient $R^2=0.9953$, slope=0.1402 and intercept=−0.1409. From this equation, the amount of sIgA in saliva was assessed from a healthy individual, a person in early stage of the common cold, and a person who has recovered from a cold. The analysis was performed by the same protocol as to generate the calibration cureve, 40 μl of saliva was mixed with 20 μl Cy5 labeled antibody plus 4 μl of free Cy5 as an internal standard. The electropherograms are shown in Fig. 5. The top panel represents saliva from healthy individual, the middle panel from a person at an early stage of common cold, and the bottom panel from a person who just recovered from the common cold. The concentration of sIgA were calculated from the linear regression equation. The results are shown in Table 1. The saliva sIgA in the healthy individual is 9.06 μg/ml, the immunocompromised patient is −0.195 μg/ml, the reason is due to the high interception value from the linear regression equation and the person who recovered from the common cold was 9.15 μg/ml. Nevertheless, the complex peak still appeared in the electropherogram of the sample which sIgA concentration may lower than 1 μg/ml. These results indicate that saliva sIgA significantly changes depending on differing status in immunity, and also indicate that this method is sensitive for detecting these changes. Possible clinical applications of this assay include prevention of respiratory tract infections or shortening duration of illness by assessing current immunity status.

Capillary based on-column immunocompetitive assay has been applied to many trace analytes, such as hormones [13], digoxin [10–12], neurotransmitters [14] and recombinant proteins [15]. In this report, we found that using a small amount of Cy5 labeled antibody to detect saliva sIgA resulted in excellent sensitivity, and may provide the opportunity for clinical use.

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References

[1] T.B. Tomasi, New Engl. J. Med. 7 (1972) 500.
[2] H. Ben-Aryeh, M. Fisher, R. Szargel, D. Laufer, Arch. Oral Biol. 35 (1990) 929.
[3] D.J. Smith, M.A. Taubman, J.L. Ebersole, J. Dent. Res. 66 (1987) 451.
[4] M.T. Ventura, Allergol. Immunopathol. (Madr.) 19 (1991) 183.
[5] J. Kugler, M. Hess, D. Haake, J. Clin. Immunol. 12 (1992) 45.
[6] R.I. Gregory, D.E. Kim, J.C. Kindle, L.C. Hobbs, D.R. Lloyd, J. Periodontal. Res. 27 (1992) 176.
[7] M.S. Ruan, Chung-Hua-Kou-Chiang-Hsueh-Tsa-Chin 25 (1990) 158.
[8] J.B. Jemmott 3rd, K. Magiloire, J. Pers. Soc. Psychol. 55 (1988) 803.
[9] J. Kugler, Psychother. Psychosomat. Med. Psychol. 41 (1991) 232.
[10] D. Schmalzing, W. Nashabeh, X.W. Yao, R. Mhatre, F.E. Regnier, N.B. Afeyan, M. Fuchs, Anal. Chem. 67 (1995) 606.
[11] F.T. Chen, S.L. Pentoney Jr., J. Chromatogr. A 680 (1994) 425.
[12] L. Tao, R.T. Kennedy, Anal. Chem. 68 (1996) 3899.
[13] Y.C. Wang, P. Su hang, X.X. Zhang, W.B. Chang, Anal. Chem. 73 (2001) 5616.
[14] A.L. Feed, J.D. Cooper, M.I. Davies, S.M. Lunte, J. Neurosci. Methods. 109 (2001) 23.
[15] E. Ban, H.S. Nam, Y.S. Yoo, J. Chromatogr. A 924 (2001) 337.